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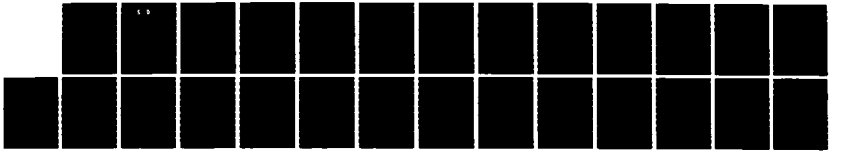

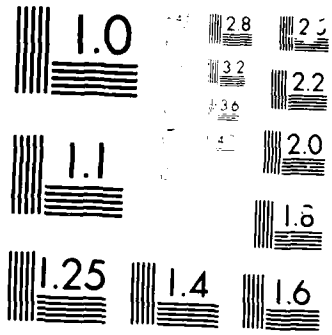
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The work supported by AFOSR was concerned with the mechanisms used by brain cells to change their functional inter-connections and the possibility that these are also involved in neuropathology. Three inter-related questions were studied: (1) what effects are produced in target neurons by prolonged exposure to neurotransmitters (e.g., acidic amino acids, acetylcholine); (2) does partial degradation of the submembrane cytoskeleton by calcium-activated proteases (calpain) affect synaptic organization; (3) are second messenger systems with known effects on growth (trophic) responses in peripheral tissues activated by intense physiological events in brain. Partial answers to each of these questions have been obtained. (1) Prolonged exposure of brain slices to acidic amino acid transmitters causes functional desensitization of "extra-synaptic" receptors, an increased binding by certain classes of receptors, and a very potent inhibition of the second messenger system normally activated by cholinergic receptors. (2) Experiments concerned with synaptic structural proteins (brain spectrin or fodrin) and a proteolytic enzyme (calpain) that digests them produced the

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22a. NAME OF RESPONSIBLE INDIVIDUAL
Dr. William O. Berry

22b. TELEPHONE (Include Area Code)
(202) 767-5021

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NL

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19. ABSTRACT

following results: a) degradation of spectrin by calpain changes irreversibly amino acid receptors; b) calpain is concentrated in synaptic regions; c) calpain-spectrin interactions in a test system (red blood cells) result in pronounced morphological changes; d) spectrin is rapidly synthesized, inserted into membrane domains, and apparently digested by calpain; d) the calpain-fodrin interaction is accelerated by calmodulin. (3) Seizures elicited by focal lesions in hippocampus cause a very rapid induction of ornithine decarboxylase, an enzyme that is activated by growth inducing stimuli in many bodily tissues. Additional studies revealed that polyamines, the synthetic products of ornithine decarboxylase, lower the set point to which brain mitochondria buffer free calcium.

RESEARCH OBJECTIVES

Brain cells receive and transmit information to each other through synaptic interactions and for a variety of theoretical reasons it is generally assumed that local changes in this process are responsible for the storage of information. There is also reason to suspect that disturbances in synaptic actions produce certain forms of neuropathology; thus excessive excitation of acidic amino receptors appears to cause cell death in many brain areas (Filer et al., 1979) while drugs (e.g. some nerve gases) that prolong the actions of acetylcholine result in pathology, at least in the periphery (Salpeter et al., 1982). Accordingly, one of the fundamental goals of neurobiology is to discover how long-lasting structural/chemical changes are produced in target cells by putative transmitters and to identify the intermediate chemistries responsible for those changes. Work in this and other laboratories over the past decade has provided some clues about the nature of these processes:

- Brief periods of intense physiological activity produce very stable changes (i.e. lasting for weeks) in synaptic transmission (Bliss and Lomo, 1973).
- These are accompanied by structural changes in synapses (Lee et al, 1980; 1981).
- The same patterns of activity also change the characteristics of receptors to the probable transmitter used in the synaptic systems exhibiting potentiation (Lynch et al,1982).
- The synaptic potentiation effect is blocked by intracellular injections of compounds that buffer calcium (Lynch et al., 1983).
- Calcium applied to synaptic membrane fractions irreversibly alters receptors (Baudry and Lynch, 1979; Baudry et al., 1983).
- The effects of calcium appear to involve the activation of a proteolytic enzyme (calpain) that degrades proteins that form the cytoskeleton lying beneath the synapses (Baudry et al., 1981; Siman et al, 1984).
- There is evidence that the same enzyme produces pathology in neurons and muscles (see Salpeter et al., 1982).

These results describe one system that begins with physiology and ends with long-term modifications of the type expected for a substrate of memory - moreover, a possible cause of neuropathology has been identified. Part of the work supported by the AFOSR has been concerned with testing various aspects of the hypothesis that physiological activity activates calpain in dendritic spines by eliciting a local increase in calcium concentrations and that calpain produces stable modifications of synaptic ultrastructure. At the same time an effort has been made to develop a more general picture of the possible ways through which intense activity might promote transient and permanent changes, including those involved in pathological reactions. This latter group of studies subdivided the general problem into two parts: 1) the possibility that transmitter accumulation, which can be expected to occur during intense activity, changes receptors or the second messenger systems that receptors activate; 2) studies on a second messenger system that is known to be involved in growth and genomic expression in systems outside the CNS, namely the enzyme ornithine decarboxylase (ODC) and the polyamines it synthesizes.

STATUS OF THE RESEARCH

1. Effects of acidic amino acid transmitters

a) Functional desensitization of two classes of receptors produced by repeated applications of acidic amino acid transmitters to hippocampal slices.

This work has been described in previous technical reports and papers; only a summary will be provided here. It might be noted that our discovery of functional desensitization has been replicated by other groups (Lewis et al., 1985; Zorumski and Fischbach, 1985).

The first experiments used extracellular electrodes to record synaptic and antidromic responses in the field CA1 of the in vitro hippocampus in the presence and absence of drugs added to the perfusion lines. A variety of acidic amino acids, some of which have been suggested to be neurotransmitters, were tested (glutamate, asparate, cysteine sulfinic

acid (CSA), quinolate, N-Methyl-D-Aspartate (NMDA), homocysteate, and kainate). All of these reversibly depolarized the target cells as evidenced by reductions in the antidromic responses and the virtual disappearance of the synaptic responses. Upon restoration of perfusion with control medium the responses quickly returned to normal. Successive applications resulted in the appearance of a desensitization to all of the amino acids except homocysteate and kainate; that is, the slices no longer responded at all to previously effective concentrations of glutamate, etc. This desensitization was itself reversible and responses to the amino acids reappeared by about 1 hour after the last of a series of closely spaced applications. It is important to note that in no case was desensitization accompanied by any change in the synaptic responses elicited by stimulation of hippocampal fibers. Thus, when the slices were totally unresponsive to glutamate, etc., the potentials produced by activation of synapses were essentially normal. This strongly suggests that the desensitized receptors were different from the transmitter receptors.

These results divided excitatory amino acid receptors in hippocampus into two groups; densitizing and non-desensitizing. By combining these categories with those reached on the basis of pharmacological experiments, we arrived at four classes of receptors; the characteristics of one of these, that which binds homocysteate, closely resemble those of the receptor for the endogenous synaptic transmitter.

These conclusions were re-examined using a completely different experimental paradigm involving the biochemical measurement of sodium fluxes in slices of hippocampus. All of the amino acids which cause physiological excitation also produce an increase in sodium flux across membranes; however, the responses elicited by several were substantially reduced by lowering the calcium concentration in the medium or by removing large numbers of axon terminals, suggesting that the amino acids in this group operate presynaptically to release neurotransmitter. Homocysteate's actions were unaffected by removal of calcium or denervation, indicating again that this amino acid operates on a postsynaptic site. Together with the earlier results, these findings strongly suggest that

homocysteate acts on an endogenous post-synaptic transmitter receptor and that this receptor does not desensitize.

Intracellular recording was then used to compare desensitizing and non-desensitizing excitatory amino acids with the changes produced by comparable treatment with carbachol, a cholinergic agonist. We replicated the earlier pattern of results for the acidic amino acids and demonstrated that desensitization occurred without any changes in resting membrane potential, membrane impedance, excitatory post-synaptic potentials (EPSP's), or inhibitory post-synaptic potentials (IPSP's). This provides strong evidence that the effect is due to a classical desensitization of an extra synaptic receptor. Carbachol (1 min. perfusion) produced a depolarization of the cells that was accompanied by an increase in membrane resistance; this pattern has been found for cholinergic agonists in several systems and is thought to reflect the closing of a potassium channel linked to a muscarinic receptor (the "M" channel). Interestingly, Cole and Nicholl (1983) argue that this is also the primary post-synaptic response elicited by stimulation of the septohippocampal fibers.

(b) Effects of prolonged exposure to acidic amino acid transmitters on receptor binding

These studies asked if prolonged exposure of slices or synaptic membrane fractions to glutamate or analogues in concentrations that might occur during transmission (0.1 to 10 mM) would influence binding properties thought to be related to synaptic receptors. Glutamate and homocysteic acid produced a four-fold increase in Na-independent, chloride-dependent binding while NMDA and kainate were without effect. This pattern of results does not match the functional desensitization observed in the physiological studies, presumeably because the receptors being assayed in the two experiments are different. The newly induced binding sites appear to be identical with pre-existing Cl-dependent binding sites by several criteria: they have a similar pharmacological profile, both are sensitive to low concentrations of Na^+ and the number of sites can be further increased by transient exposure to micromolar calcium concentrations. Moreover, binding of ^{3}H-APB, a ligand

selective for the Cl-dependent glutamate binding sites, is also increased after glutamate pre-incubation. The induction of binding sites by high glutamate concentrations is calcium independent, not inhibited by leupeptin and, therefore, different from the previously described activation of binding sites by a calcium sensitive protease. The high concentrations of ligand needed to induce increased binding suggests the presence in hippocampal membranes of a binding site with low, millimolar affinity that is functionally related to the known high-affinity binding sites (Kessler et al., 1986).

Following upon these results, we carried out additional physiological studies in which slices were exposed to glutamate analogues, and in particular homocysteic acid, for longer periods than was the case in our first studies (section 1 above). These prolonged applications produced a loss of synaptic physiology probably because of the known cytotoxic effects of acidic amino acids. However, APB which occupies a glutamate binding site and is chiefly an antagonist produced a very large increase in synaptic potentials when applied in high concentrations and then removed from the slices. This effect did not reverse after an hour of washout.

Summarizing the experiments with acidic amino acids we find that brief applications desensitize extra-synaptic but not synaptic receptors, that prolonged applications cause an increase in binding to what may be synaptic receptors and pathophysiology, and that receptor occupancy without intense depolarization leads to increase in binding and stable physiological modifications. There may be a continuum of effects in which transmitter accumulation first reduces extra synaptic receptors, modifies synaptic receptors and physiology, and finally causes cell damage.

(c) Effects of excitatory amino acids on a second messenger system activated by acetylcholine

The above results provide a first description of how accumulation of putative acidic amino acid transmitters influences various classes of receptors and synaptic physiology. The next studies in the series investigated the possibility that prolonged exposure might influence the chemistry of other receptor systems, including those for acetylcholine and

norepinephrine. Recent studies from a number of laboratories have shown that these transmitters (as well as several hormones) activate a potent second messenger system involving increased turnover of membrane phosphoinositol with the production of two intracellular messengers, inositol triphosphate (IT) and diacylglycerol (DG). IT and DG promote the release of calcium from intracellular stores and activate protein kinase C; there is reason to suspect that this system produces cell-wide changes associated with growth in many bodily tissues (Berridge and Irvine, 1984). The functions of the PI system in brain are unknown although there is increasing evidence that kinase C activation alters physiological channels associated with potassium (Baraban et al., 1985) - given its effects in other tissues, it seems likely that the PI system will be found to have broader, "trophic" effects as well. Our studies revealed that excitatory amino acids have little if any direct effect on phosphoinositol turnover that they profoundly inhibit the activation of the system by acetylcholine (and histamine) but not that elicited by norepinephrine. This effect was receptor mediated since it was blocked by appropriate antagonists and was not due to the depolarization produced by the excitatory amino acids since it was not reproduced by potassium-induced depolarization (Baudry et al., 1986).

It appears then that activation of synaptic and extrasynaptic acidic amino acid receptors somehow alters the relationship of the cholinergic receptor to PI turnover and thereby disrupts a potent second messenger system. This raises the possibility that the long-term changes produced by prolonged exposure to these compounds is mediated by the interruption of a normally present trophic influence.

2. Proteases and Structural Proteins

There is now a compelling body of evidence showing that membranes of most and perhaps all types are superimposed over a cytoskeletal matrix, a key constituent of which is a protein known as spectrin (Goodman and Zagon, 1985). Spectrin is linked to actin filaments and tubulin on the one side and to the membrane by anchoring proteins on the other. This matrix serves to limit the mobility of transmembrane proteins and very probably governs the surface properties and shape of cells. Our discovery that spectrin in

brain and red blood cells is a substrate protein for a calcium activated protease (calpain) and that this interaction resulted in an irreversible change in glutamate binding (Baudry et al., 1981; Siman et al., 1984) provided one plausible candidate for the mechanism through which physiological activity could produce long-term biochemical and structural changes at synapses. It also suggested a link between plasticity and pathology since there is good evidence that calpain activation causes degeneration in peripheral nerves and muscles (Schlaepfer and Hasler, 1979). There are a number of important issues concerning calpain and brain spectrin that remain unanswered and recently funds from the AFOSR grant have been used to investigate some of these.

(a) Localization of calpain

It has been known for some years that calpain I found in brain (Guroff, 1964; Murachi et al., 1981; Simonson et al., 1985) but its regional and subcellular distributions had not been studied. Subcellular fractionation experiments in our laboratory have produced a most surprising result: the low threshold form of calpain (i.e., that variant activated by low micromolar concentrations of calcium) are found primarily in the soluble fractions of synaptosomes (a brain fraction containing budded-off axon terminals and dendritic spines). Table I summarizes the data pertinent to this point ((Baudry, DuBrin, and Lynch, in prep.). Further study reveals that calpain "translocates" in the presence of low concentrations of calcium from the soluble compartment into the membranes where it autodigests itself, as well as breaking down spectrin, until calcium is removed (figure I; Seubert, Baudry, DuBrin, and Lynch, unpublished).

Immunocytochemical experiments have been done in parallel with this work using monoclonal antibodies directed against purified calpain (Siman et al., 1985). We now have electron microscopic data on the enzyme's subcellular location in hippocampal pyramidal cells; these show that calpain is primarily post-synaptic, and is localized to post-synaptic densities, spines, and to the tubule system in the core of the dendrite (Perlmutter et al., in prep.). These results together with the biochemical data suggest that intense physiological activity and transmitter accumulation should first activate calpain in the region of

the synapse (by causing calcium influx); if the calcium influx were sufficiently large, then enzyme should translocate from the soluble compartment onto the membranes producing further breakdown of brain spectrin. Prolonged elevation of calcium could lead to activation of calpain in the microtubules and thus dendritic pathology.

(b) Effects of calpain activation in red blood cells

It is very difficult to analyze the effects of calpain activation on structure in the CNS - and for this reason we have used red blood cells to gain information on the morphological effects produced by the enzyme (Siman et al., 1986a). It might be noted that other groups using blood platelets have obtained strong evidence in favor of our hypothesis that calpain uncovers receptors (Baldassare et al., 1985). Red blood cells (RBC) undergo a marked physical transformation from a flat smooth profile (discocyte) to an elaborate rounded form (echinocyte) when their internal calcium levels are elevated. Our studies show that this effect is correlated with a breakdown of spectrin, blocked by inhibitors of calpain and is mimicked in RBC "ghosts" when calpain is added to the interior of the cell (Siman et al., 1986a; Table II). These results indicate that calpain activation can produce rapid, dramatic, and irreversible modifications of structure.

(c) Further studies of the calpain-brain spectrin interaction

An understanding of how calpain-spectrin interactions might regulate the structure and chemistry of synapses will ultimately require information about the extent to which calpain regulates the concentration of the structural protein. That is, we might imagine that the postulated plasticity mechanism is simply an exaggeration of a normally present, low rate process; or, alternatively, it could represent an exotic event occurring only in unusual circumstances. There is indirect evidence for the former idea. Lazarides and co-workers (Woods and Lazarides, 1985) have shown that erythocyte spectrin exists in a rapidly turning over soluble pool and a more stable membrane-associated compartment. We have found that brain spectrin is also found in soluble and membrane-linked pools; and that the concentration of the former is highly and negatively ($r = 0.90$) correlated with total calpain activity. Inhibitors of calpain infused into the brain cause a detectable

increase in the concentration of soluble brain spectrin pool (Siman, Atwood, and Lynch, 1986). There is also evidence for calpain degradation of brain spectrin in membranes and post-synaptic densities. Carlin et al. (1983) have reported high concentrations of a spectrin fragment of 150,000 daltons in highly purified post-synaptic densities and we have shown that this same immunologically identified breakdown product is produced by calpain activation (Siman et al., 1984).

Recent studies done in collaboration with Wolfram Bodsch using his technique for measuring protein synthesis in the brain suggests that the degradation of spectrin into this breakdown product occurs continuously at a slow but measurable rate; specifically radio-labelled, immunologically identified spectrin breakdown product appears within six hours of the time that native spectrin is synthesized. Interestingly enough, this fragment appears in both soluble and membrane bound fractions (Bodsch, Baudry and Lynch, in prep., see figure II). Taken together these experiments provide evidence that the calpain-spectrin interaction is a continuous process and thereby increase the likelihood that local exaggerations (e.g., in synaptic areas experiencing large fluxes of calcium) are not uncommon.

Finally, we have recently obtained direct evidence that the calpain spectrin interaction in brain, with the concommitant appearance of the 150,000 dalton breakdown product, can be greatly accelerated by local conditions. Spectrin contains a calmodulin binding site which until now has had no known functional significance. As shown in figure III, calmodulin causes a four to six fold increase in the rate at which spectrin is degraded by calplain. This effect is completely blocked by trifluoroperazine, a calmodulin antagonist, and is not accompanied by any increase in calpain activity. Thus calmodulin at concentrations known to exist in the brain somehow modifies spectrin such that it is more readily digested by calpain (Seubert, Baudry and Lynch, in prep.). Calmodulin is known to be released from neuronal membranes during intense depolarization - the above results provide a route through which spine depolarization might act synergistically with calcium influx to promote the breakdown of structural proteins. We are currently explor-

ing the functional capacities of the 150,000 dalton breakdown product - it may be that calpain modifies synaptic operation by changing the relative balance of native and partially digested spectrin.

3. Polyamines, Calcium, and Trophic Influences

The above experiments were concerned with local (i.e. synaptic) modulations due to alterations in synaptic communication. It is widely accepted that neurons also exert trophic influences upon each other such that (unknown) modulatory substances released from one cell affect gene experession in a second (for a review, see Black et al., 1984). It is presumeably against the background of these very long term interactions that plasticity and pathology inducing conditions exert their effects. Recently, Dr. Christine Gall (U.C.I. Anatomy Department) discovered a paradigm that provides a means for testing how physiological events and transmitter accumulation exert trophic effects and the consequences these might have for those cellular agencies concerned with the regulation of neuroplasticity. Briefly, she found that seizures produced by focal lesions in hippocampus dramatically alter the balance of neuropeptides in a defined synaptic system (Gall et al., 1981) and that this is due to perturbations in gene expression (White et al, 1986). In a collaborative effort, we tested the possibility that these alterations are preceded by an induction of ornithine decarboxylase (ODC), an enzyme that synthesizes polyamines. The rationale for this idea is simple: ODC induction is known to precede and indeed to be responsible for growth responses in a large number of peripheral tissues in a variety of experimental conditions. Our experiments indicated that focal seizures cause a fifty-fold increase in ODC activity and that this takes place several hours before enhanced peptide production occurs (Baudry et al., 1986). The polyamines synthesized by ODC produce a host of effects but, as might be appreciated from the description of the studies on calpain, our first interest is with their possible effects on calcium buffering. Specifically, it has been reported that polyamines cause a marked increase in calcium buffering by mitochondria in hepatocytes (Nicchitta and Williamson, 1984). The only high capacity calcium buffering device available to neurons resides in the mitochondria (Bygrave, 1977) and any

major alterations in this machinery can be expected to produce significant changes in the calcium buffer, particularly during periods of intense physiological activity. Accordingly, we reinvestigated the affect of polyamines on calcium uptake by brain mitochondria and found the same marked enchancement reported for hepatocytes (Jensen, Baudry, and Lynch, submitted).

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Table I: Distribution of calpain I and II in various subcellular fractions for rat telencephalon.

Fraction	Calpain I	Calpain II
Homogenate	0.32 ± 0.22	6.61 ± 1.42
Soluble (S_2)	4.08 ± 0.65	27.2 ± 2.5
Synaptosomes	17.5 ± 2.1	1.25 ± 0.71
Mitochondria	2.48 ± 0.80	0.

Calpain activity in different subcellular fractions was measured using ^{14}C-casein as a substrate. Results are expressed as dpm/μg protein/hr and are means ± S.E.M. of 4-6 experiments.

Table II. Effects of various incubation conditions on shape change and band 3 proteolysis in erythrocyte ghosts.

ADDITIONS	% DISCOCYTES	BAND 3 REMAINING (% CONTROL)
No Additions	95	100
Calcium, 10 μM	35	57
, 30 μM	18	32
, 100 μM	7	18
Calcium, 100 μM		
+ Leupeptin, 3 μM	50	45
, 30 μm	83	86
+ Antipain, 5 μM	59	48
, 50 μM	95	91
+ Iodoacetate, 5 mM	78	87
+ N-Ethylmaleimide, 5 mM	62	73
Pepstatin A, 500 μM	10	17
+ PMSF, 1 mM	8	12
+ Trifluoperazine, 50 μM	5	12

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Siman, R., Ahdoot, M. and Lynch, G. Ontogeny, compartmentation and turnover of spectrin isoforms in rat central neurons. J. Neurosci. 1986. (Submitted)

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In preparation :

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Perlmutter, L., Gall, C., Baudry, M., Siman, R. and Lynch, G. Ultrastructural localization of Calpain I in rat hippocampus. Brain Res.

Seubert, P., Baudry, M. and Lynch, G. Calmodulin stimulates the degradation of brain spectrin by calpain. J. Cell Biol.

PROFESSIONAL PERSONNEL (1983 - present)

M. Baudry	Associate Professor (in residence), Psychobiology, UCI
L. Fagni	post-doctoral fellow (now at CNRS)
M. Kessler	research psychobiologist
R. Siman	post-doctoral fellow (now at E. I. DuPont)
C. Gall	Assistant Professor (Anatomy Department, UCI)
J. Jensen	graduate student (Ph.D. expected December 1986)
L. Perlmutter	post-doctoral fellow, UCI
P. Seubert	post-doctoral fellow, UCI

FIGURE LEGENDS

Figure I. Effect of calcium on the binding of calpain I to rat brain SPMs.

Purified calpain I was incubated with increasing concentrations of calcium in the presence of rat brain SPMs. Free and bound calpain were separated by centrifugation; membrane-bound calpain was re-extracted by incubating SPMs with 1mM EGTA and 0.1% Triton X-100 followed by centrifugation. Calpain activity was determined using ^{14}C-casein as a substrate.

Figure II. In vivo turnover of brain spectrin in synaptosomal fraction of rat brain.

^{35}S-Methionine was slowly perfused in the lateral ventricle under anesthesia for various periods of times. Animals were sacrificed by decapitation and various subcellular components were obtained by differential centrifugation; following separation of Triton-soluble and insoluble proteins, brain spectrin (alpha and beta - subunits as well as the breakdown product, bdp) were immunoprecipitated and quantitated.

Figure III. Effect of calmodulin on calpain-induced brain spectrin degradation.

Purified brain spectrin was incubated with purified calpain I (from rat erythrocyte) in the presence and absence of calmodulin (5 μM) and with or without trifluoroperazine (TFP, 50 μM). At the indicated times, reaction was stopped by adding a solubilization solution, proteins were separated on SDS polyacrylamide slab gels and the remaining content of spectrin determined by quantitative densitometry of the Coomasie Blue stained gels.

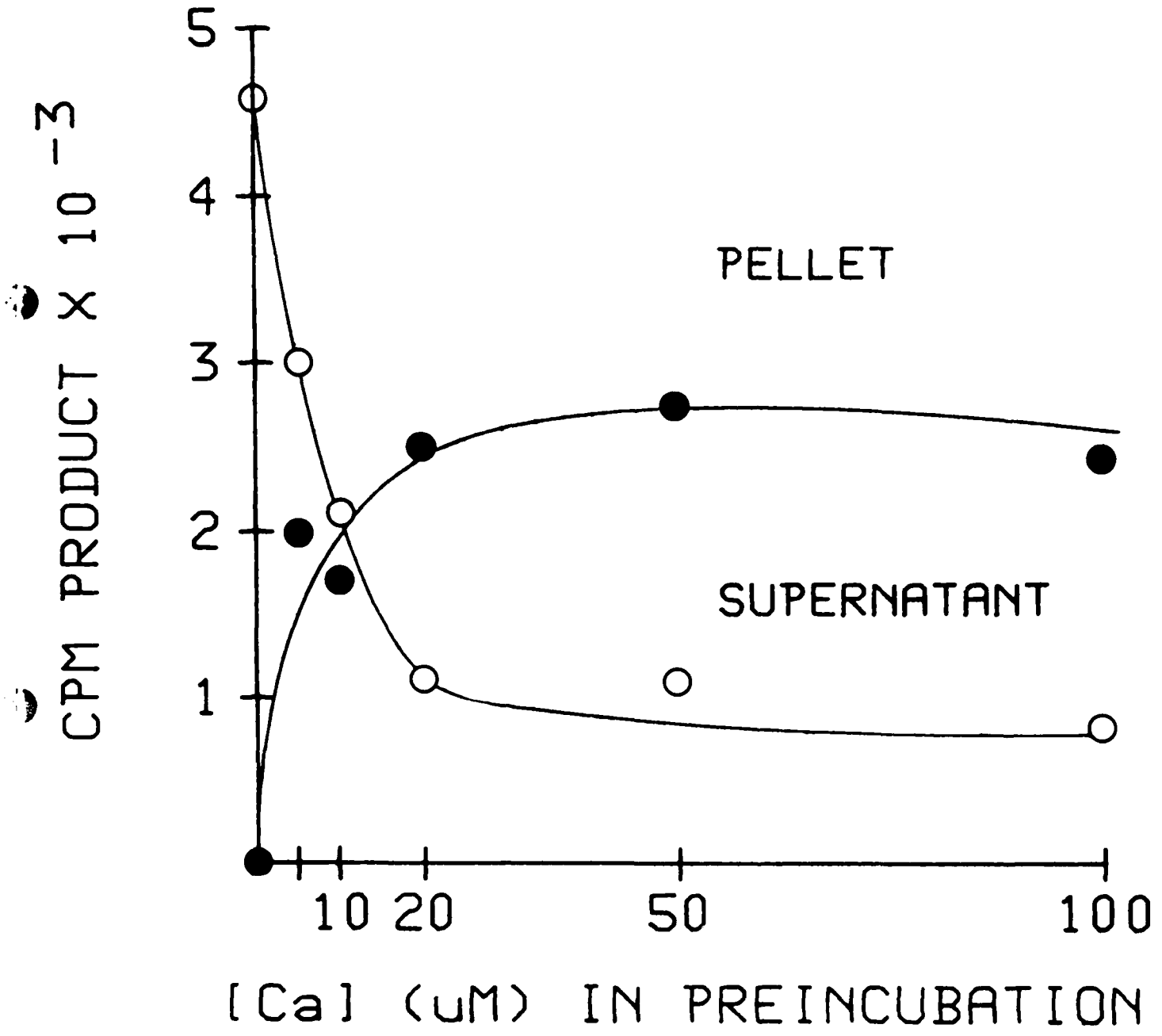
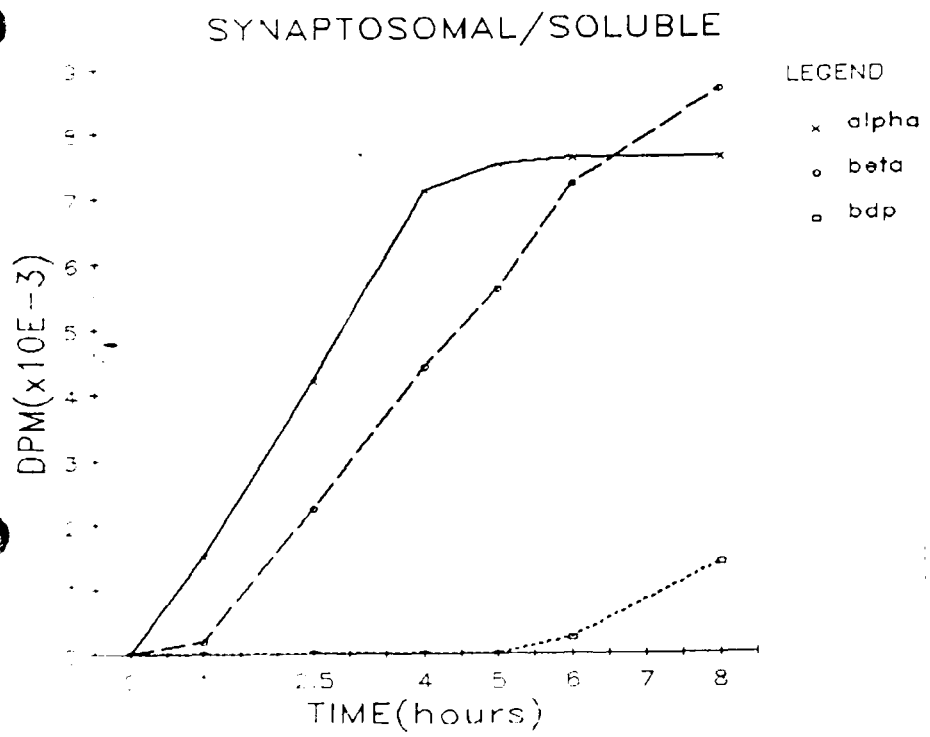
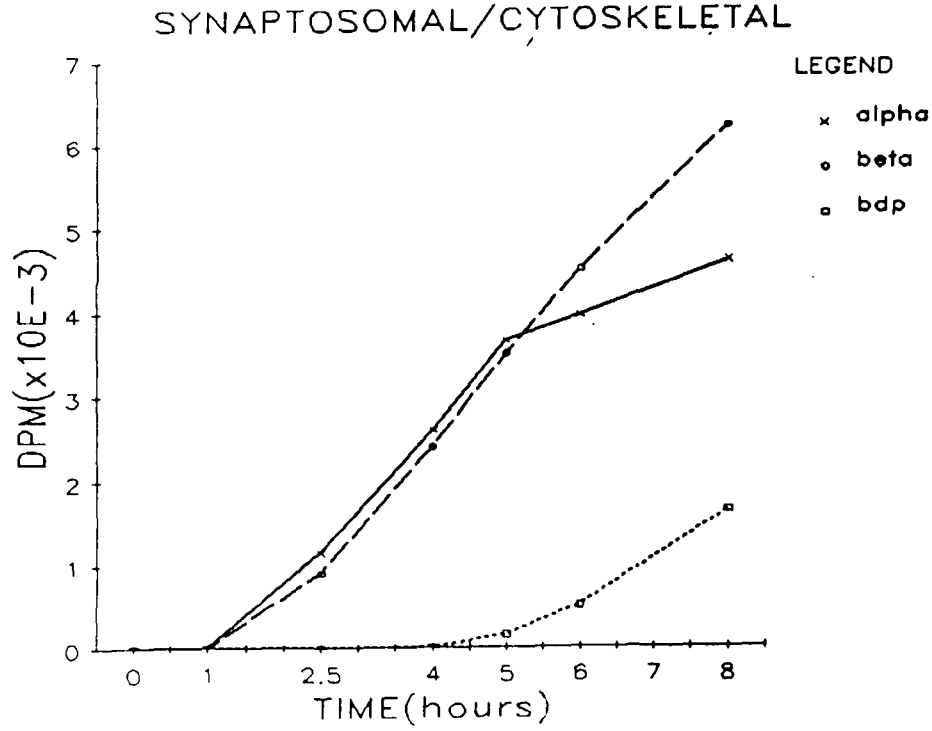

CPM PRODUCT X 10 -3
5
4
3
2
1
PELLET
SUPERNATANT
10 20
50
100
[Ca] (uM) IN PREINCUBATION

FIG. 1


SYNAPTOSOMAL/SOLUBLE
DPM(x10E-3)
TIME(hours)
LEGEND
alpha
beta
bdp


SYNAPTOSOMAL/CYTOSKELETAL
DPM(x10E-3)
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1
2
3
4
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8
TIME(hours)
LEGEND
alpha
beta
bdp

Fig 2

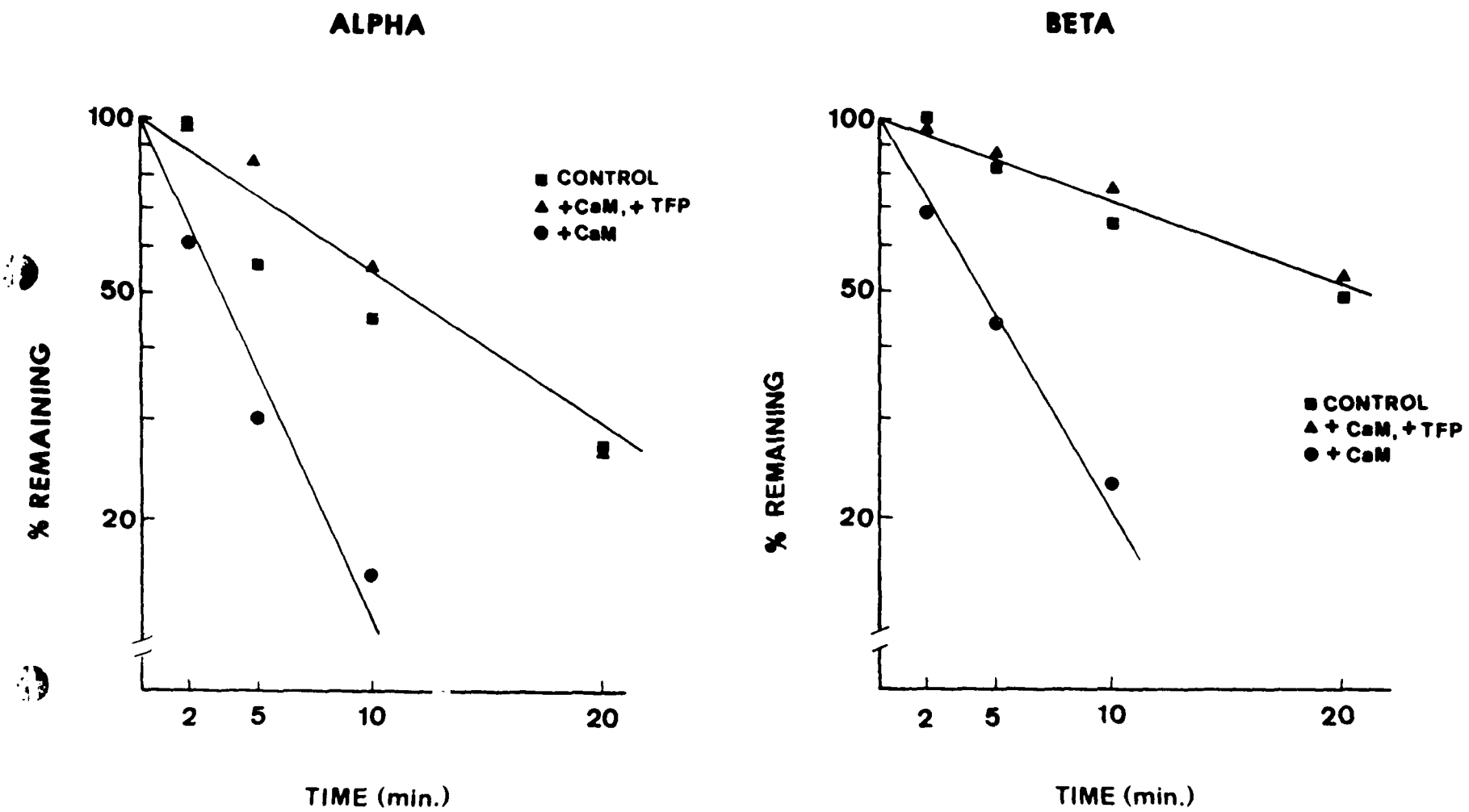
ALPHA
100
50
20
% REMAINING
CONTROL
+CaM, + TFP
+CaM
2
5
10
20
TIME (min.)
BETA
100
50
20
% REMAINING
CONTROL
+ CaM, + TFP
+ CaM
2
5
10
20
TIME (min.)

Fig 3

END

DTIC

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